Original Article

Exosomal circMACF1 drives PI3K/AKT/mTOR-mediated autophagy suppression in laryngeal squamous cell carcinoma

Yingnan Zhu, Chuanxin Duan, Yunzhi Gui, Danxia Chen, Xi Su*

Department of Otolaryngology, Maternal and Child Health Hospital of Hubei Province, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430070, China

Article Info

Abstract

Laryngeal squamous cell carcinoma (LSCC) is a common malignant tumor. The regulatory functions of circular RNAs (circRNAs) in cancers have been broadly reported. The hsa_circ_0011773 (circMACF1) is reported to be overexpressed in LSCC tissues, while its biological function in LSCC remains unclear. CircMACF1 expression in LSCC tissues and cells was assessed via RT-qPCR. Exosomes extracted from cells were identified by TEM and NTA. Autophagy-related proteins were tested by western blot. Confocal microscope was employed for analyzing LC3 expression. Cell proliferation, migration, and invasion were assessed by CCK-8 assay and transwell assay. The levels of main proteins on PI3K/AKT/mTOR were tested by western blot. We observed that circMACF1 was highly expressed in LSCC tissues and cells. Furthermore, circMACF1 expression was also upregulated in the exosomes derived from LSCC cells. CircMACF1 depletion promoted LC3 expression in cells. Additionally, we proved that circMACF1 knockdown suppressed LSCC cell proliferative, migratory and invasive capabilities via promoting autophagy. Exosomal circMACF1 was found to promote LSCC tumor growth. Then, we proved that circMACF1 could activate PI3K/AKT/mTOR pathway to regulate autophagy. Moreover, MACF1 was positively regulated by circMACF1 and its overexpression notably reversed the effects of circMACF1 depletion in LSCC progression. Exosomal circMACF1 can regulate PI3K/AKT/mTOR-mediated autophagy suppression to facilitate LSCC development.

Keywords: Laryngeal squamous cell carcinoma, CircMACF1, Exosomes, Autophagy, PI3K/AKT/mTOR pathway

1. Introduction

Laryngeal squamous cell carcinoma (LSCC) is one of the most common malignant tumors in head and neck tumors [1]. Heavy drinking, smoking and Human Papilloma Virus infection are considered as the risk factors for LSCC [2]. Due to the concealment of the laryngopharyngeal cavity, the early symptoms in patients are not obvious and are very easy to ignore [3]. Additionally, LSCC has a strong aggressiveness, so the mortality rate of the disease is high, up to about 50%. At present, the common treatment methods for LSCC include surgery, chemotherapy and radiotherapy [4]. However, despite a series of related active treatments, the survival rate of patients is still very low, and the prognosis is also poor. Thus, the exploration of the molecular mechanism of LSCC is the key to developing more effective treatment methods.

Circular RNAs (circRNAs) are the class of noncoding RNAs that commonly exist in the cytoplasm of assorted eukaryotic cells [5]. Different from linear RNAs, circRNAs possess a stable circular structure without 5′ to 3′ polarity. Accumulating evidence has identified the dysregulation of circRNAs in various human cancers and proved their biological function in cancers, including LSCC.

For example, circ_0110389 upregulation facilitates cell growth of gastric cancer by modulating SORT1 [6]. Circ_0000043 accelerates cell proliferative and migratory capabilities and epithelial-mesenchymal transition (EMT) process in breast cancer through regulating Smad3 [7]. Circ_0001421 overexpression contributes to glycolysis and tumor growth in lung cancer [8]. Circ_0067934 can predict the poor prognosis and facilitate LSCC cell proliferation via sponging miR-1324 [9]. A study has shown that plasma-derived hsa_circ_0011773 is significantly overexpressed in LSCC tissues, implying circ_0011773 may act as a potential biomarker for predicting LSCC [10]. The host gene of circ_0011773 is MACF1, so circ_0011773 was named circMACF1 in this study. Thus, the biological function of circMACF1 in LSCC was explored.Exosomes are the small membrane vesicles (around 30-150 nm in diameter) secreted by most cells [11]. They widely exist in blood, urine, and saliva [12]. Exosomes that contain non-coding RNA, mRNAs, and proteins exert vital function in intercellular communication via transferring their contents, including proteins and nucleic acids, to receptor cells [13]. Importantly, exosomes from tumor can be secreted in the tumor environment, thus affecting tumor

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growth, metastasis, angiogenesis and drug resistance. Exosome-derived circTRPS1 is reported to facilitate cell malignant phenotypes in bladder cancer [14]. Cancer-derived exosomal circ_0038138 expedites glycolysis and metastasis of gastric cancer through the miR-198/EZH2 axis [15]. CircMACF1 comes from plasma, suggesting that circMACF1 may be mediated by exosome.

Thus, this study aimed to investigate the function and mechanism of exosomal circMACF1 in LSCC. This study may offer a new regulatory mechanism for understanding the pathogenesis of LSCC.

2. Materials and methods
2.1. Tissue samples

25 paired LSCC and adjacent non-cancer tissues were obtained from patients who underwent radical resection in Maternal and Child Health Hospital of Hubei Province, Tongji Medical College, Huazhong University of Science and Technology. The tissues were immediately stored at -80 °C. All patients provided written informed consent. None of them received chemoradiotherapy prior to surgery. The blood samples were gathered, and plasma was acquired through centrifugation. We have received approval from the Ethics Committee of Maternal and Child Health Hospital of Hubei Province, Tongji Medical College, Huazhong University of Science and Technology.

2.2. Cell culture

LSCC cells Tu177 (Bioleaf Biotech Co., Ltd, Shanghai, China) and TU212 (BNCC, Beijing, China) as well as the human bronchial epithelioid cell 16-HBE (BNCC) were utilized for this study. Cells were incubated in DMEM (Invitrogen, Carlsbad, CA, USA) and added with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2.

2.3. Cell transfection

For silencing circMACF1 expression, the shRNA for circMACF1 (sh-circMACF1; 30 nM) and the negative control (sh-NC; 30 nM) were synthesized by GenePharma (Shanghai, China). For overexpressing circMACF1 or MACF1, the full-length sequence of circMACF1 or MACF1 was inserted into the pcDNA3.1 vector (Geenseed Biotech, Guangzhou, China). Cell transfection was performed through the Lipofectamine 3000 (Invitrogen) for 48 h in line with user guides.

2.4. RT-qPCR

Total RNAs from cells or tissues were isolated by 1 mL of TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). Then, the cDNA was synthesized by Reverse Transcription kit (GenePharma), and the qPCR was conducted by SYBR Green PCR kit (Takara, Dalian, China) on StepOne Plus System. Gene expression was measured via the $2^{-\Delta\Delta Ct}$ method. GAPDH served as the control.

2.5. Western blot

The extracted proteins were lysed by RIPA, followed by isolating via 10% SDS-PAGE and transferring to the PVDF membranes. The membranes were blockaded by 5% nonfat milk for 2 h and incubated with the following primary antibodies (Abcam, USA) at 4°C for one night. HRP-conjugated secondary antibody (Abcam) was utilized to further incubation of 2 h. Protein bands were tested by the ECL kit and analyzed by ImageJ.

2.6. Actinomycin D and RNase R assay

Cells were treated with 2 mg/ml of Actinomycin D (ActD; Sigma-Aldrich, St. Louis, MO, USA) or DMSO as the control. For RNase R assay, cells were treated with 3 U/μg RNase R for 1 h. RT-qPCR was applied for analyzing gene expression.

2.7. Fluorescence In Situ Hybridization (FISH)

The circMACF1-specific FITC-labeled probes were applied for performing FISH assay. Nuclei were dyed by DAPI and assays were implemented in accordance with the kit protocols (GenePharma).

2.8. Subcellular fractionation assay

The isolation of cytoplasmic and nuclear circMACF1 in 1×10^6 cells were achieved by PARIS™ Kit (Invitrogen) as per the guidebook. After purification, isolated RNAs (circMACF1, GAPDH, U6) were analyzed through RT-qPCR.

2.9. CCK-8 assay

Cells were seeded in 96-well plates at a density of 2000 cells per well. Cells were incubated with 10 μl CCK-8 solution (Dojindo, Japan) at the indicated time for another 2 h. The absorbance at 450 nm was assessed by the microplate reader (Molecular Device, USA).

2.10. Colony formation assay

Cells were plated into 6-well plates for incubation for 14 days. Afterwards, they were fixed in 4% polyformaldehyde for 10 min and then dyed with 0.5% crystal violet for half an hour, followed by analysis with a microscope (Olympus, Japan).

2.11. Transwell assay

Cell migration and invasion were detected by transwell chamber (8μm pore size, Corning). The 5×10^4 cells in serum-free culture medium were added to the upper chamber. For invasion assay, the upper chamber was precoated with Matrigel (10ug/ml; BD Biosciences, USA). Culture medium including 10% FBS was supplemented to the lower chamber for 48 h. After that, cells in the lower chamber were fixed and dyed. A light microscope (Olympus) was utilized for observation.

2.1.2. Exosome isolation and identification

Exosomes were isolated from cells utilizing a Hieff™ Quick Exosome Isolation Kit (Yeasen, Shanghai, China). The cell fragments and dead cells from medium were removed through centrifugation at 2500 × g for 10 min. Next, 10 ml medium was gathered into a centrifuge tube and extraction reagent was supplemented. Then they were mixed and was allowed to stand for 2 h. Later, the medium was subjected to centrifugation at 10000 × g at for 1 h. The precipitates were then subjected to re-suspension in PBS. Next, the solution was transferred to the EP tube and subjected to centrifugation at 12000 × g for 2 min. In the end, the supernatant comprising exosomes was gathered.

Exosome identification was carried out through Transmission Electron Microscope (TEM; JEOL, Japan) for observing morphology. The marker proteins were determined via western blot. The particle size of exosomes was tested via Nanoparticle Tracking Analysis (NTA) through ZetaView PMX 110 (Particle Metrix, Germany) and Ori-

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ginPro 8.5.

2.1.3. Confocal imaging

Cells expressing LC3B tagged with GFP were employed for detecting autophagy through confocal fluorescence microscopy. Cells expressing the tandem mCherry-LC3 construct were performed. Cells expressing GFP-LC3 or mCherry-LC3 and transfected with the indicated plasmids were incubated in a glass-bottomed chamber for one night, followed by replacement with DMEM-containing starvation media. Nuclei were dyed by DAPI. LSM780 confocal microscope (Carl Zeiss, Germany) was applied for imaging and the quantification was performed by ZEN black software (Carl Zeiss).

2.1.4. Animal experiment

This experiment was approved by the Ethics Committee of Maternal and Child Health Hospital of Hubei Province, Tongji Medical College, Huazhong University of Science and Technology. BALB/c nude mice (male; 22–25 g; 6-8 weeks old) were purchased from Maternal and Child Health Hospital of Hubei Province, Tongji Medical College, Huazhong University of Science and Technology. The exosomes were isolated from the transfected TU177 cells and named sh-NC-exo or sh-circMACF1-exo. Mice were subjected to subcutaneous injection with TU177 cells (3×10^6) stably circMACF1 or exosomes into the left side. After 28 days, mice were euthanized. Subsequently, the tumor was removed and weighed.

2.1.5. Immunohistochemical (IHC) analysis

The paraffin-embedded tumor tissues were cut into 5-μm slices, and then deparaffinized in xylene and rehydrated by fractional ethanol. Then slices were repaired by antigen and blocked with goat serum. After that, they were incubated with the primary antibody against Ki67, mTOR, or p-mTOR (Abcam) at 4 °C for one night. Afterwards, slices were incubated with secondary antibody (Abcam). Next, they were dyed by DAB solution for 5 min. The light microscope (Olympus) and Image-Pro Plus 6.0 software were utilized for analysis.

2.1.6. Immunofluorescence (IF) staining

After deparaffinization and rehydration, tissues were incubated with EDTA and rinsed with PSB, followed by blocking with goat serum. Next, they were cultured with the LC3-II antibody at 4°C for one night. FITC-labeled secondary antibody was supplemented for further incubation of 1 h. The sections were sealed with neutral resins and analyzed by the laser-scanning confocal microscope (Olympus).

2.1.7. Statistical analyses

Statistical analysis was performed by GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). The difference between groups was analyzed by Student's t-test or one-way ANOVA. Data are displayed as the mean $\pm$ SD from three individual repeats. $P < 0.05$ represented statistical significance.

3. Results

3.1. CircMACF1 is upregulated in LSCC tissues and cells

Firstly, we studied circMACF1 expression in LSCC. RT-qPCR outcomes illustrated that circMACF1 was upregulated in the serum and tissues of LSCC patients relative to the normal controls (Figure 1A-B). In comparison to 16-HBE, circMACF1 was significantly expressed in LSCC cells (TU177 and TU212) (Figure 1C). In order to detect the circular feature of circMACF1, we started a series of experiments. The cyclization of circSLC1A4 was verified by agarose gel electrophoresis, and we discovered circMACF1 can only be amplified in cDNA via adopting divergent primers but not gDNA (Figure 1D). The RNase R assay manifested that circMACF1 expression in the cells treated with RNase R was almost unchanged, while MACF1 mRNA expression was almost completely digested (Figure 1E). RT-qPCR further detected the expression of circMACF1 and MACF1 mRNA in cells before and after treatment with Actinomycin D. As a result, circMACF1 was more stable than linear MACF1 mRNA expression (Figure 1F). These experimental results confirmed the circular characteristics of circMACF1 in LSCC cells. In addition, the subcellular localization of circMACF1 was further estimated. FISH and subcellular assays demonstrated that circMACF1 was expressed in both the nucleus and cytoplasm of LSCC cells (Figure 1G-H). In conclusion, we confirmed the high expression of circMACF1 in LSCC tissues and cells, indicating that circMACF1 may participate in LSCC development.

3.2. Exosomal circMACF1 inhibits autophagy of LSCC cells

Autophagy exerts a vital function in the genesis and progression of LSCC, so we tested the regulatory role of circMACF1 on autophagy of LSCC cells. Firstly, we knocked down circMACF1 expression by transfecting sh-circMACF1 plasmids into cells (Figure 2A). Next, we examined the effect of silencing circMACF1 on the expression of autophagy-associated protein LC3 and mTOR. LC3-II/
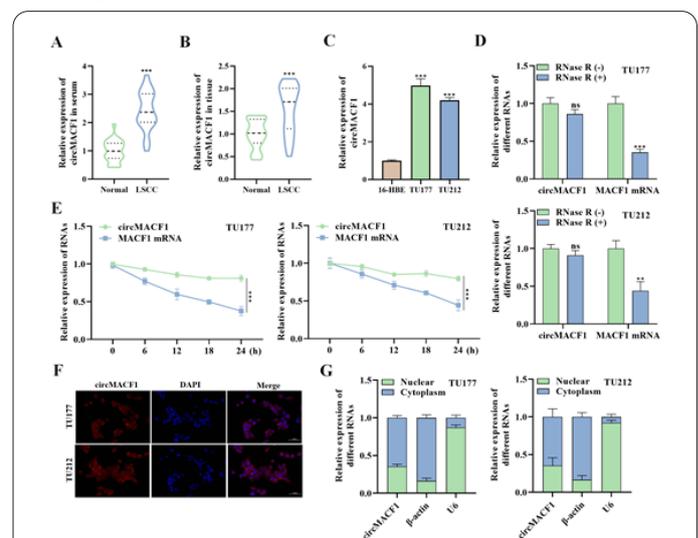

Fig. 1. CircMACF1 is highly expressed in LSCC tissues and cells. (A-B) RT-qPCR outcomes of circMACF1 expression in the serum and tissues of LSCC patients or normal patients. (C) RT-qPCR outcomes of circMACF1 expression in TU177, TU212, and 16-HBE cells. (D) Agarose gel electrophoresis was utilized to determine the circular feature of circMACF1 in cells. (E-F) CircMACF1 and MACF1 expression in cells treated with RNase R or Actinomycin was tested through RT-qPCR. (G-H) FISH assay and subcellular location assay were applied to determine the position of circMACF1 in cells. **p<0.01, ns: no significance.

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LC3-I ratio is an important indicator of autophagy activation. Activation of mTOR can inhibit autophagy. Western blot results showed that circMACF1 silencing notably elevated LC3-II/LC3-I ratio and declined mTOR protein level, indicating the activation of autophagy (Figure 2B-C). Furthermore, through confocal microscope, we discovered that the LC3 puncta numbers were notably increased by circMACF1 silencing, suggesting autophagy flux was enhanced by circMACF1 downregulation (Figure 2D). In previous experiments, we confirmed that circMACF1 was highly expressed in serum of LSCC patients, which suggested that circMACF1 might be mediated by exosomes. Thus, we carried out relevant experiments to verify it. We found that there was no obvious alteration in circMACF1 expression in the cells added with RNase A relative to the control. However, in the cells added with RNase A and Triton X-100, we observed a significant decrease in the expression of circMACF1, suggesting that circMACF1 could be mediated by exosomes (Figure 2E). Through TEM, we observed that the shape of exosomes was small and round (Figure 2F). NTA detection further displayed the diameter range of exosomes (Figure 2G). Western blot results manifested that CD63, CD81, and TSG101 were positive for exosomes (Figure 2H). RT-qPCR results showed that circMACF1 expression in TU177-exo and TU212-exo was significantly increased compared with 16-HBE-exo (Figure 2I). Next, we found that sh-circMACF1-exo significantly increased the ratio of LC3-II/LC3-I and decreased the protein level of mTOR (Figure 2J-K). Overall, these results demonstrated that the exosomal circMACF1 could inhibit the autophagy of LSCC cells.
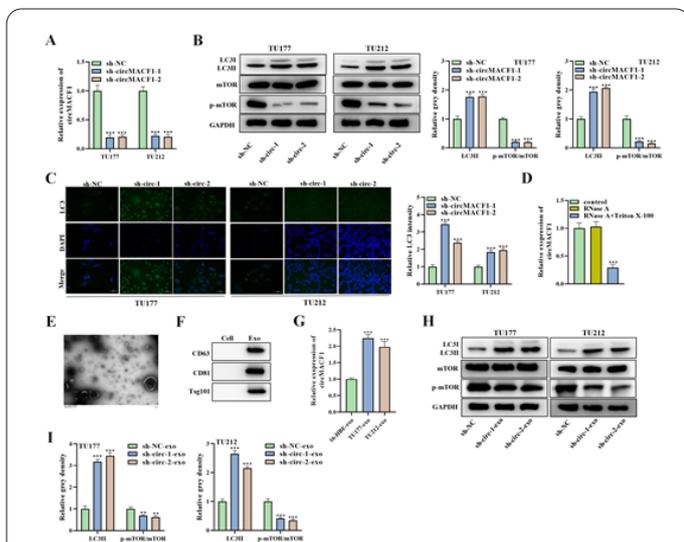

Fig. 2. Exosomal circMACF1 inhibits autophagy of LSCC cells. (A) RT-qPCR outcomes of the transfection efficiency of sh-circMACF1 in cells. (B-C) Western blot outcomes of LC3, mTOR, and p-mTOR levels in cells after circMACF1 was silenced. (D) Confocal microscope analysis showed the LC3 expression in cells after circMACF1 was silenced. (E) RT-qPCR outcomes of circMACF1 in cells treated with RNase A and Triton X-100. (F) TEM image of exosomes. (G) NTA analyzed the particle diameter of exosomes. (H) Western blot outcomes of CD63, CD81, and TSG101 levels in cells and exosomes. (I) RT-qPCR outcomes of circMACF1 expression in 16-HBE-exo, TU177-exo, and TU212-exo. (J-K) Western blot outcomes of LC3, mTOR, and p-mTOR levels in cells transfected with sh-circMACF1-exo or sh-NC-exo. **p<0.01.
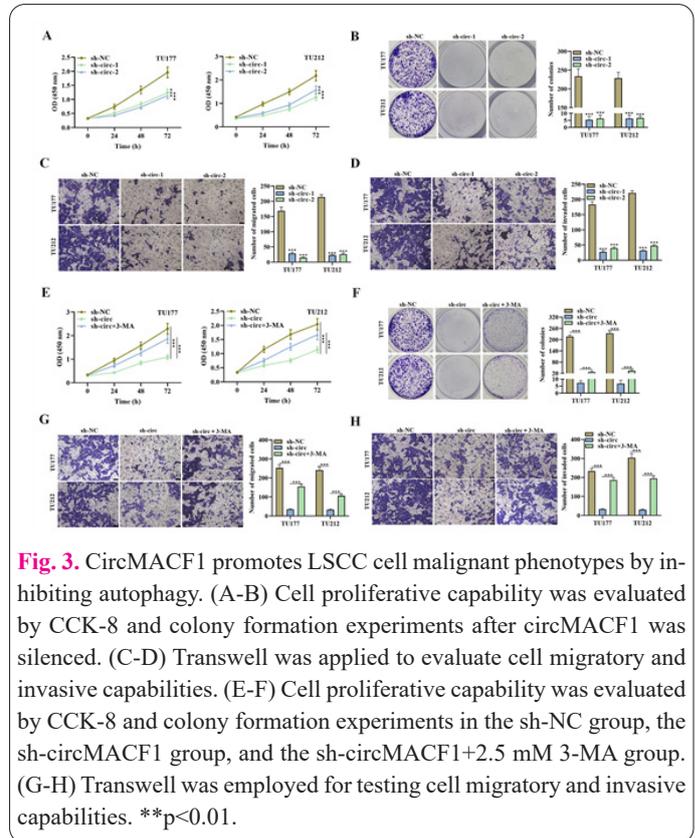

Fig. 3. CircMACF1 promotes LSCC cell malignant phenotypes by inhibiting autophagy. (A-B) Cell proliferative capability was evaluated by CCK-8 and colony formation experiments after circMACF1 was silenced. (C-D) Transwell was applied to evaluate cell migratory and invasive capabilities. (E-F) Cell proliferative capability was evaluated by CCK-8 and colony formation experiments in the sh-NC group, the sh-circMACF1 group, and the sh-circMACF1+2.5 mM 3-MA group. (G-H) Transwell was employed for testing cell migratory and invasive capabilities. **p<0.01.

3.3. CircMACF1 facilitates LSCC cell proliferation, migration and invasion via suppressing autophagy

We used functional assays to assess circMACF1 function on the behaviors of LSCC cells. The results of CCK-8 experiment manifested that after interference with circMACF1, the absorbance of OD450 nm was decreased significantly, indicating the inhibition of cell proliferation (Figure 3A). Colony formation assay further confirmed that circMACF1 deletion reduced the number of cell colonies (Figure 3B). Transwell assays demonstrated that circMACF1 knockdown significantly reduced the number of migrating cells and invasive cells (Figure 3D). However, the inhibitory function of circMACF1 knockdown on cell proliferation, migration and invasion were notably reversed by 3-MA (autophagy inhibitor) supplement (Figure 3E-H). Therefore, circMACF1 promoted LSCC cell behaviors via inhibiting autophagy.

3.4. Exosomal circMACF1 promotes tumor growth in LSCC

We further tested the effect of circMACF1 on tumor growth through *in vivo* experiments. As shown in Figure 4A-C, compared with the nude mice injected with sh-NC, the tumor morphology in the nude mice injected with sh-circMACF1 was significantly reduced, and the tumor volume and weight notably declined. Additionally, IHC results showed that Ki67, mTOR and p-mTOR expression in tumor tissues of the sh-circMACF1 group was significantly decreased compared with sh-NC group (Figure 4D). IF assay further indicated that the fluorescence point of LC3 in sh-circMACF1 group was markedly increased (Figure 4E). In addition, we injected the sh-NC-exo or sh-circMACF1-exo into mice subcutaneously for observing the effect of exosomal circMACF1 on tumor growth of LSCC. We discovered that the tumors in sh-circMACF1-exo group were significantly smaller, and the tumor volume and weight were notably lower (Figure 4F-H). Fur-

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thermore, the expression of Ki67, mTOR, and p-mTOR as well as the LC3 fluorescence intensity in tissues was reduced by the injection of sh-circMACF1-exo in mice (Figure 4I-J). These results verified that exosomal circMACF1 repressed autophagy and promoted the growth of LSCC tumor in mice.

3.5. CircMACF1 promotes LSCC progression and activates PI3K/AKT/mTOR pathway by upregulating MACF1

CircRNAs have been confirmed to regulate cancer progression through its host genes [16, 17]. Therefore, we studied whether circMACF1 can promote LSCC development by regulating MACF1. Firstly, we found that circMACF1 knockdown notably suppressed MACF1 levels (Fig. 5A-B). Then, the results of functional experiments showed that the cell proliferation repressed by circMACF1 knockdown was restored by MACF1 overexpression (Fig. 5C-D). We continued to observe that the migratory and invasive capabilities were weakened by circMACF1 downregulation, but MACF1 overexpression improved these abilities (Fig. 5E-F). Finally, circMACF1 downregulation was observed to suppress the activity of PI3K/AKT/mTOR signal pathway and promote LC3-II/LC3-I ratio, but this result was reversed by the overexpression of MACF1 (Fig. 5G-H). The above results can prove that circMACF1 promoted LSCC progression by upregulating MACF1 and activating PI3K/AKT/mTOR pathway.

4. Discussion

At present, the treatment strategy for LSCC cannot effectively improve the survival rate and prognosis of patients [4]. Therefore, the development of new targeted therapeutic strategies is of great significance. CircRNAs are covalently closed RNA with a closed loop and were
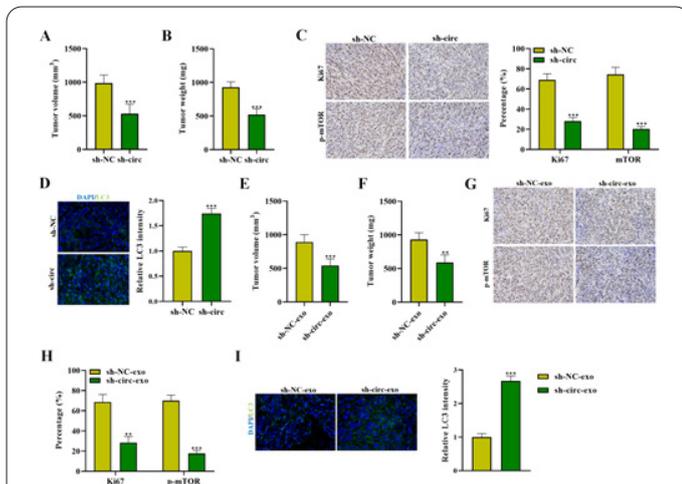

Fig. 4. Exosomal circMACF1 promotes the development of LSCC tumor. (A) Schematic diagram of tumor morphology in nude mice of the sh-NC group and the sh-circMACF1 group. (B-C) The tumor volume and weight of mice were determined. (D) IHC results of Ki67 and mTOR expression in tumor tissues in different groups. (E) IF was utilized to detect LC3 expression in tissues. (F) Schematic diagram of tumor morphology in mice. (G-H) The tumor volume and weight in the sh-NC-exo group and the sh-circMACF1-exo group were determined. (I) IHC results of Ki67 and mTOR expression in the sh-NC-exo group and the sh-circMACF1-exo group. (J) IF was utilized to detect LC3 expression in the sh-NC-exo group and the sh-circMACF1-exo group. **p<0.01.
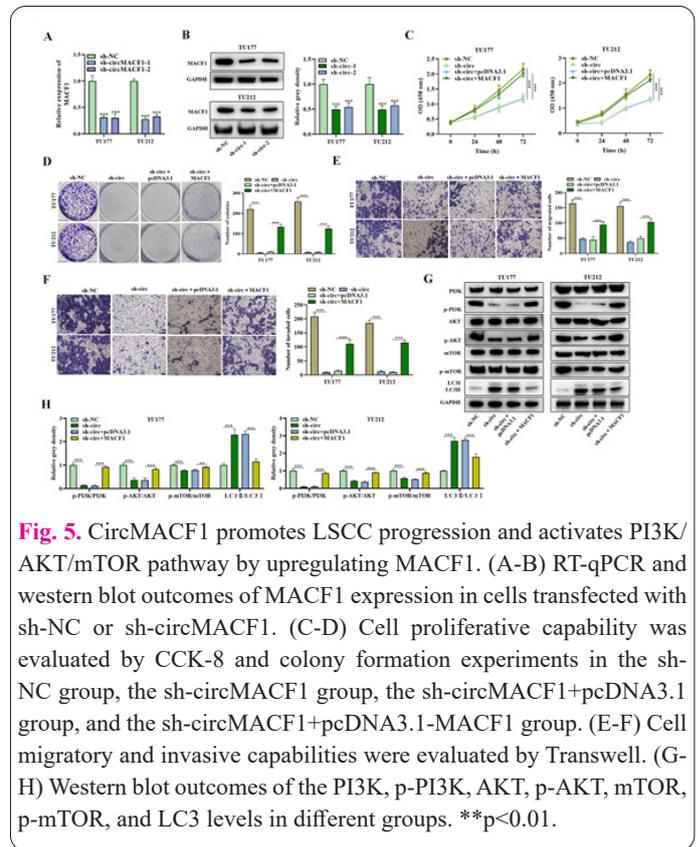

Fig. 5. CircMACF1 promotes LSCC progression and activates PI3K/AKT/mTOR pathway by upregulating MACF1. (A-B) RT-qPCR and western blot outcomes of MACF1 expression in cells transfected with sh-NC or sh-circMACF1. (C-D) Cell proliferative capability was evaluated by CCK-8 and colony formation experiments in the sh-NC group, the sh-circMACF1 group, the sh-circMACF1+pcDNA3.1 group, and the sh-circMACF1+pcDNA3.1-MACF1 group. (E-F) Cell migratory and invasive capabilities were evaluated by Transwell. (G-H) Western blot outcomes of the PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR, and LC3 levels in different groups. **p<0.01.

active in modulating the expression of genes. Accumulating studies have confirmed that circRNAs exert a vital function in tumor development. Furthermore, some circRNAs have been identified to participate in the progress of LSCC, such as circ_0120175 [18], circ_0044520 [19], and circ_0003221 [20]. In this study, we found that circMACF1 was reported to be highly expressed in LSCC and may become a potential biomarker [10]. However, there is very little functional research on circMACF1. Zhao et al. have suggested that circMACF1 can attenuate acute myocardial infarction via targeting the miR-500b-5p-EMP1 axis [21]. Fan et al. suggest that the circ_MACF1/miR-942-5p/TGFBR2 axis can modulate drug sensitivity in non-small cell lung cancer [22]. In this study, we proved that circMACF1 was notably overexpressed in serum and tissues of LSCC patients. CircMACF1 upregulation in LSCC cell lines was also verified. *In vivo* and *in vitro* assays proved that circMACF1 can promote cell proliferation, migration, invasion, and tumor growth in LSCC, suggesting that circMACF1 acts as an oncogene in LSCC. CircRNA enrichment in cancer-derived exosomes has been confirmed by lot of studies [23]. Furthermore, circRNAs have been confirmed to be more easily enriched in exosomes relative to linear RNA. In this study, we extracted the exosomes from LSCC cells and discovered that circMACF1 displayed an overexpression in exosomes. Furthermore, exosomal circMACF1 could facilitate tumor growth in mice. Thus, we confirmed that exosomal circMACF1 derived from cancer cells can regulate LSCC development.

Autophagy exerts the vital function in tumorigenesis and development. Autophagy is a self-degradative progression that is crucial for balancing sources of energy in development and responding to nutrient stress [24]. In this process, the cytoplasm and cell proteins are swallowed, digested, and then recycled to maintain cell metabolism.

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Studies have revealed that autophagy is a double-edged sword in cancer progression due to the diversity of cells, conditions and stimulators. On the one hand, autophagy has been proven to inhibit the growth of primary tumor and promote the apoptosis of cancer cells. On the other hand, autophagy can inhibit cell apoptosis and is a necessary factor to maintain tumor development. In addition, autophagy also promotes tumor metastasis by regulating cell migration, invasion and EMT progress [25]. Circ-SPECC1 regulates autophagy to accelerate hepatocellular carcinoma growth and metastasis [26]. It has been reported that inhibition of autophagy is a promising therapeutic strategy for laryngeal cancer [27, 28]. CircPARD3 facilitates cell proliferation and migration of LSCC through suppressing autophagy [29]. Similarly, herein, we proved that circMACF1 silencing increased LC3-II/LC3-I ratio and decreased mTOR protein levels, indicating the activation of autophagy. Furthermore, in rescue assays, we discovered that the addition of autophagy inhibitor 3-MA obviously reversed the impact of circMACF1 silencing on cell malignant phenotypes in LSCC. *In vivo* assays demonstrated that exosomal circMACF1 could repress autophagy and facilitate tumor growth in LSCC mice. Thus, we confirmed that circMACF1 facilitated LSCC progression by autophagy inhibition.

The PI3K/AKT/mTOR signaling pathway is the main pathway modulating autophagy, which could regulate cell survival and apoptosis [30, 31]. The importance of upregulation and activation of this pathway in cancers has been widely studied. Circ_0010882 was reported to facilitate gastric cancer development through the PI3K/AKT/mTOR pathway [32]. Circ-PRKDC suppresses autophagy and apoptosis in T-cell acute lymphoblastic leukemia via microRNA-653-5p-mediated PI3K/AKT/mTOR pathway [33]. CircPARD3 promotes LSCC development via suppressing autophagy by the PRKCI-Akt-mTOR pathway [29]. Nevertheless, it is not clear whether circMACF1 affects PI3K/AKT/mTOR pathways in LSCC. Herein, circMACF1 knockdown notably could suppress the key proteins on PI3K/AKT/mTOR pathway, indicating that circMACF1 activated this pathway in LSCC. Furthermore, LY294002 treatment reversed the effect of circMACF1 depletion on the pathway activity and LC3 expression. Collectively, we confirmed that circMACF1 activated PI3K/AKT/mTOR pathway to suppress autophagy in LSCC.

The mechanism of circRNA in cancer by regulating its host gene has been reported in many studies [16, 17]. MACF1 is a member of spectraplakin family. MACF1 exerts the crucial function in regulating cell proliferation, embryo development, signal transduction, and maintenance of tissue integrity. Accumulating researches have revealed that MACF1 is involved in assorted human cancers. For example, MACF1 promotes melanoma metastasis via suppressing EMT process. MACF1 is correlated with predicted poor prognosis in breast cancer and has been confirmed as a gene responsible for breast cancer [34]. MACF1 facilitates acute myeloid leukemia cell growth via upregulating Runx2 and activating the PI3K/Akt signaling [35]. Similarly, we proved MACF1 expression could be decreased via circMACF1 silencing in LSCC cells. Rescue assays confirmed that MACF1 overexpression reversed the suppressive functions of circMACF1 depletion in LSCC cell proliferative, migratory, and invasive capabilities. Furthermore, MACF1 upregulation activated the PI3K/AKT/mTOR signaling and inhibited autophagy in LSCC.

5. Conclusion

Taken together, this study proved that exosomal circMACF1 drives PI3K/AKT/mTOR-mediated autophagy suppression to regulate cell malignant phenotypes and tumor growth in LSCC. These discoveries may provide the novel therapeutic targets for LSCC.

Conflict of Interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
We have received approval from the Ethics Committee of Maternal and Child Health Hospital of Hubei Province, Tongji Medical College, Huazhong University of Science and Technology.

Informed Consent
We have received informed consent from the Ethics Committee of Maternal and Child Health Hospital of Hubei Province, Tongji Medical College, Huazhong University of Science and Technology.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
ZY conducted the experiments and wrote the paper; DC, GY and CD analyzed and organized the data; SX conceived, designed the study and revised the manuscript.

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